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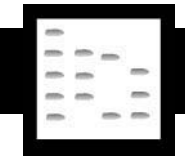
ECDYSTEROIDS: CHROMATOGRAPHY



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Introduction

Ecdysteroids are present both in animals (mainly Arthropods) and plants and comprise about 300 different molecules related to ecdysone (**Figure 1**). Structural variation in the number of carbons on the side-chain and of substituents at various positions (**Table 1**) results in the presence of compounds displaying very different polarities. Most available chromatographic techniques have been applied to the isolation and analysis of ecdysteroids. Paper chromatography is now obsolete and no longer used. Gas chromatography (GC) is of limited use, as the derivatization procedures necessary to make volatile derivatives require careful control. Currently, the most widely used techniques are high performance liquid chromatography (HPLC) and thin-layer chromatography (TLC), with the former providing the major analytical methods.

Liquid–Liquid Partitions

The simplest separation method concerns partitioning between two non-miscible solvents, and it is currently used for clean-up of biological samples. On this basis, several procedures have been designed, which allow the preparation of almost pure compounds in the gram scale.

Solvent Partitioning

Partition between n-butanol and water can be used to remove polar contaminants, whereas partition between aqueous methanol and hexane removes non-polar materials. Lipids can also be removed from aqueous extracts with hexane–methanol (7 : 3, v/v), light petroleum or n-propanol–hexane (3 : 1, v/v). The nature of the contaminants to be removed and that of the ecdysteroids to be isolated govern the choice for a given partition system. The number of free -OH groups significantly affects partition coefficients (**Table 2**).

The combination of two successive partition steps allows the elimination of both polar and apolar contaminants. It is thus possible to combine (1) chloroform/water and (2) water/butanol. This results in a butanol-containing fraction that is significantly enriched. It is possible to select a narrower range of polarity by replacing chloroform with a more polar organic solvent, e.g. isobutyl acetate, that nevertheless allows ecdysteroids to remain in the water phase.

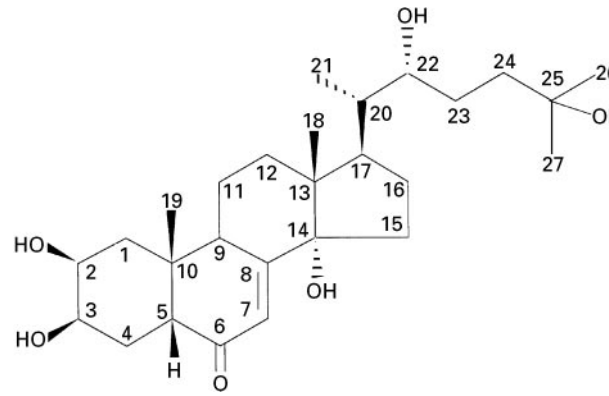

Figure 1 The structure of ecdysone, the first ecdysteroid isolated from *Bombyx mori* pupae.

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Table 1 Variations on the 20-hydroxyecdysone molecule

Type	*Positions on the molecule*
Hydroxyl groups	
Additional –OH	1, 5, 11, 16, 18, 19, 23, 24, 26
Missing –OH	2, 20, 22, 25
Oxidation	
(>CHOH → >C = O)	3, 22
(–CH_2OH → –COOH)	26
(→ epoxide)	22–23
Epimerization	3α/3β, 5α/5β
Alkyl substitution	24 (methyl, methylene, ethyl, etc.)
Esterification	
Acetates	2, 3, 22, 25
Fatty acyls	22
Benzoates	20, 22, 25
Cinnamates	2
Coumarates	3
Phosphates	2, 22, 26
Sulfates	22
Lactone ring formation	Concerns mainly C-28 or C-29 ecdysteroids
Etherification	
Intramolecular	Between C-22 and C-25
Methoxy ether	25
Ketal/acetal formation	
Acetonides	2–3, 20–22
Benzylidene acetals	20–22
Glycosylation	
Galactosides	3, 22
Glucosides	3, 22, 25, 26
Dehydration	9(11), 14(15), 24(25), 25(26)
Side-chain cleavage	C-20/C-22, C-17/C-20

Counter-current Distribution

Counter-current distribution (CCD) is a multi-tube extension of the above partition procedure. Butenandt and Karlson (1954) purified the first ecdysteroid (ecdysone) from *Bombyx* pupae by CCD with butanol–cyclohexane–water (4 : 6 : 1). This technique is presently of limited use, and it has been replaced by the more convenient droplet counter-current chromatography technique described below.

Droplet Counter-current Chromatography

Droplet counter-current chromatography (DCCC) allows an efficient purification of crude samples up to the gram range (**Table 3**). DCCC enables the preparation of reasonably, although not absolutely, pure compounds (a subsequent HPLC step may be required to get pure ecdysteroids). One DCCC separation usually lasts several days: exchanges between mobile droplets and the stationary phase is the rate-limiting process. High-speed counter-current chromatography (HSCCC) overcomes this drawback and separations are performed within a few hours. This technique has so far only been applied to the ecdysteroids in a small number of cases.

Table 2 Partition coefficients (K) of ecdysteroids (data from Lafont *et al*., 1994b)

Ecdysteroid	*K*
Cyclohexane–n-butanol–water (5 : 5 : 10)	
Ecdysone	3.54
Makisterone A	1.27
20-Hydroxyecdysone	0.52
3-Epi-20-hydroxyecdysone	0.52
26-Hydroxyecdysone	0.39
20,26-Dihydroxyecdysone	0.06
Chloroform–methanol–water (2 : 1 : 1)	
2,22-Dideoxyecdysone	13.0
2-Deoxyecdysone	2.7
Ecdysone	0.4
20-Hydroxyecdysone	0.1

$$K = \frac{\text{concentration in the non-polar phase}}{\text{concentration in the polar phase}}$$

Thin-Layer Chromatography (TLC)

Normal-phase (absorption) chromatography on silica gel has been used extensively in the isolation of ecdysteroids and for metabolic work. Despite the advent of HPLC, the low expense, simplicity and speed of TLC ensures a continuing role for this technique in ecdysone research.

Chromatographic Procedures

Normal-phase systems Many solvent systems have been used for TLC of ecdysone and related compounds, and these are summarized in **Table 4**. A wide range of R_F values on silica gel have been reported.

Table 3 Solvent systems for droplet counter-current chromatography (DCCC)

Solvent system	*Mode*
$CHCl_3$/MeOH/H_2O (13 : 7 : 4)	Ascending
$CHCl_3$/C_6H_6/EtOAc/MeOH/H_2O (45 : 2 : 3 : 60 : 40)	Descending
C_6H_6/$CHCl_3$/MeOH/H_2O (5 : 5 : 7 : 2)	Descending
$CHCl_3$/MeOH/H_2O (65 : 20 : 20)	Descending

$CHCl_3$, chloroform; MeOH, methanol; C_6H_6, benzene; EtOAc, ethyl acetate.

Table 4 Some representative solvent systems for TLC of ecdysteroids on silica gel

Solvent system	*Composition*	[R_F]	
		E	20E
$CHCl_3$/95% EtOH	7 : 3	0.39	0.34
$CHCl_3$/MeOH	9 : 1	0.10	0.07
$CHCl_3$/Pr–1–OH	9 : 5	0.21	0.12
CH_2Cl_2/Me_2CO/MeOH	2 : 1 : 1	0.69	0.62
CH_2Cl_2/Me_2CO/EtOH	16 : 4 : 5	0.32	0.10
CH_2Cl_2/MeOH/H_2O	79 : 15 : 1	0.32	0.19
CH_2Cl_2/MeOH/ 25% NH_3–H_2O/H_2O	77 : 20 : 2 : 1	0.47	0.40
EtOAc/EtOH	4 : 1	0.49	0.46

E, ecdysone; 20E, 20-hydroxyecdysone. $CHCl_3$, chloroform; EtOH, ethanol; MeOH, methanol; Pr–1–OH, n-propanol; CH_2Cl_2, dichloromethane; Me_2CO, acetone; EtOAc, ethyl acetate.

For consistent results, plates should be heated at 120°C for 1 hr, then deactivated to constant activity over saturated saline. An example of the type of separation that can be achieved in metabolic studies in insects is shown in **Figure 2**.

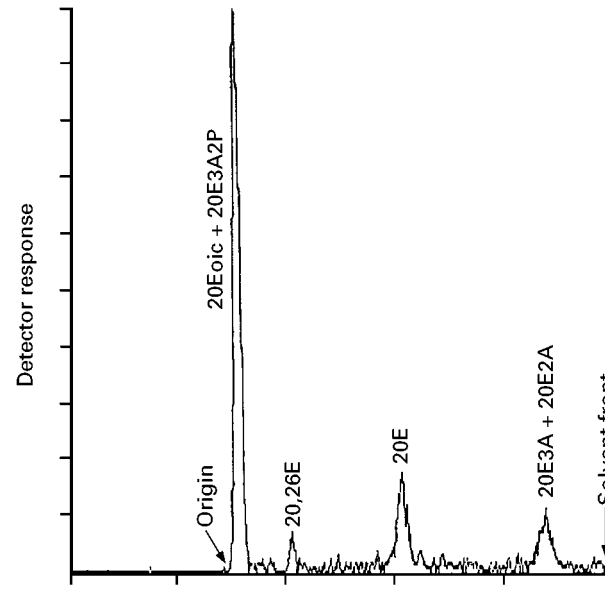

Figure 2 Normal-phase TLC on a silica gel high-performance TLC plate using chloroform–ethanol (4 : 1) to separate 20-hydroxyecdysone (20E) metabolites formed in an insect. 20E3A2P, 20-hydroxyecdysone 3-acetate 2-phosphate; 20Eoic, 20-hydroxyecdysonoic acid; 20,26E, 20,26-dihydroxyecdysone; 20E2A, 20-hydroxyecdysone 2-acetate; 20E3A, 20-hydroxyecdysone 3-acetate. After Wilson ID and Lafont R (1986). Thin-layer chromatography and high-performance thin-layer chromatography of [^{3}H] metabolites of 20-hydroxyecdysone. *Insect Biochemistry* 16: 33–40, reprinted with permission.

Reversed-phase systems An alternative to normal-phase TLC (NP-TLC) is reversed-phase TLC (RP-TLC) on silica bound to alkyl chains (2 to 18 carbons). Methanol–water, ethanol–water, isopropanol–water, acetonitrile–water and acetone–water systems have been used as mobile phases, with methanol–water solvents providing the most general solvent system. The order of migration in RP-TLC is roughly opposite of that seen using NP-TLC (**Table 5**). Results vary significantly with plate manufacturers. Changing the proportion of methanol in the solvent over the range 0 to 100% increases the R_F values of ecdysone and 20-hydroxyecdysone, and this shows a quite linear relationship between the percentage of methanol in the solvent and R_F (**Figure 3**).

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Table 5 R_F for representative ecdysteroids in TLC

Compound	*System 1*	*System 2*	*System 3*
Calonysterone	0.42	0.20	0.37
Cyasterone	0.33	0.40	0.51
2-Deoxy-20-hydroxyecdysone	0.31	0.21	0.29
2-Deoxyecdysone	0.38	0.15	0.17
Ecdysone	0.21	0.29	0.28
20-Hydroxyecdysone	0.15	0.44	0.38
20-Hydroxyecdysone 2-cinnamate	0.53	0.04	0.03
Inokosterone	0.17	0.44	0.37
Kaladasterone	0.49	0.17	0.30
Makisterone A	0.20	0.31	0.40
Muristerone A	0.27	0.32	0.31
Polypodine B	0.22	0.42	0.44
Ponasterone A	0.42	0.16	0.18
Ponasterone C	0.38	0.29	0.37
Ponasterone C 2-cinnamate	0.65	0	0
Poststerone	0.32	0.37	0.38

System 1, silicagel plates, solvent $CHCl_3$–MeOH (4 : 1); System 2, Merck C_{18}-bonded plates, solvent MeOH–H_2O (1 : 1); System 3, Whatman C_{18}-bonded plates, solvent MeOH–H_2O (1 : 1).

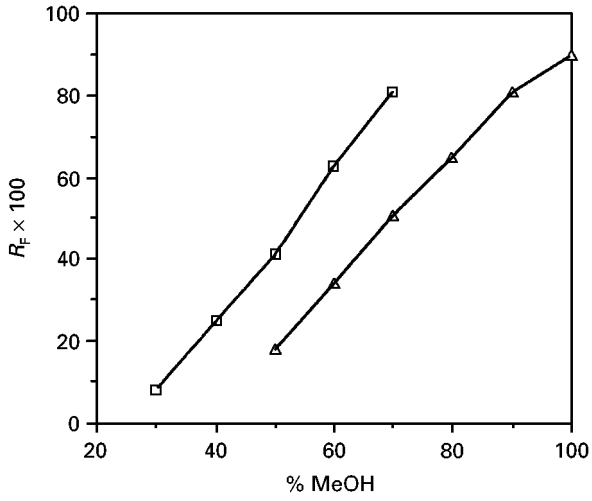

Figure 3 Influence of the solvent composition (MeOH-water) on the R_F of ecdysone and 20-hydroxyecdysone analysed by RP-TLC on C_{18}-bonded plates. □, 20-hydroxyecdysone; Δ, ecdysone.

Detection of Ecdysteroids after TLC

Detection of ecdysteroids on the TLC plate can be accomplished using a set of techniques of varying specificities (**Table 6**). Non-specific techniques include iodine vapour, heating in the presence of ammonium carbonate (which produces fluorescent spots), or fluorescence quenching if a fluorescing agent is incorporated into the silica. More specific reagents such as the vanillin–sulfuric acid spray can be used to give spots of characteristic colour. Sulfuric acid or ammonia give fluorescent reactions, with the former being slightly more specific.

Evolved TLC Techniques

Automatic multiple development (AMD) In AMD, the plate is repeatedly developed with the same solvent, which migrates more and more with each development. This method allows a reconcentration of ecdysteroids at each run, in particular by suppressing tailing, and this finally results in sharper bands and improved resolution.

Over-pressure layer chromatography (OPLC) In OPLC, the solvent is forced through the layer by an HPLC pump and the plate can be developed within minutes. The use of high-performance TLC plates rather than conventional TLC plates is recommended for optimal results. Developing the plates under these conditions will minimize diffusion, while allowing mass transfer to proceed.

Neither AMD nor OPLC have been used to any significant extent for the separation of ecdysteroids, although where they have been employed for these compounds good results have been obtained.

Low-Pressure Column Chromatography

Preparative Columns

These systems are used for the isolation and purification of ecdysteroids from crude extracts. Columns are

Table 6 Various methods for the visualization of ecdysteroids after TLC

Method	*Operating mode*
UV absorbance	Direct visualization under UV light: poorly sensitive method Use of a scanner and obtention of UV spectra
[1]**Fluorescence quenching**	Use of silica plates containing a luminescent agent (ZnSe)
Non-specific colour reactions	I_2 vapours Phosphotungstic acid gives blue colour Anisaldehyde
Fluorescence induction	H_2SO_4 $(NH_4)_2CO_3$
[1]**Vanillin spray reagent**	Spray with vanillin/95% EtOH/H_2SO_4 (5 : 70 : 25, w/v/v), then heat at 100–120°C for 10 min
Reactions for 3-oxoecdysteroids	Folin–Ciocalteu gives blue colour 2,4-Diphenylhydrazine gives yellow colour (+ $K_3Fe(CN)_6$ gives orange colour) Triphenyltetrazolium chloride gives red colour
Radioactivity	Scanner or autoradiography (metabolic studies)
Mass spectrometry	Direct introduction of the plate, or FAB-MS on scraped silica

[1]Classical methods.

Table 7 Some low pressure chromatographic systems for medium- or large-scale purification of ecdysteroids

Stationary phase	*Solvent system*
Normal phases	
Silica (silica gel, silicic acid or celite)	$CHCl_3$/MeOH (100 : 3; 95 : 5; 80 : 20; or SG[1])
	$CHCl_3$/EtOH (19 : 1)
	CH_2Cl_2/EtOH (SG)
	EtOAc/MeOH (SG)
Alumina	$CHCl_3$/MeOH (2 : 1; or SG)
	$CHCl_3$/EtOH (SG)
	CH_2Cl_2/EtOH (9 : 1; or SG)
	EtOAc/MeOH (1 : 1)
	EtOAc/EtOH (2 : 1; 1 : 1; or SG)
	$Me_2CO/CH_2Cl_2/H_2O$ (62.5 : 15 : 10)
Sephadex LH20	$CHCl_3$/EtOH (88 : 12)
	CH_2Cl_2/MeOH (SG)
	CH_2Cl_2/Me_2CO
Reversed-phases	
Amberlite XAD-2	H_2O/MeOH (SG)
	H_2O, then EtOH
Amberlite XAD-16	H_2O, then EtOH
Sephadex LH20	EtOH/H_2O (7 : 3)
	MeOH
Polyamide	H_2O
Ion-exchange	
DEAE-Sephadex	Step-gradient of NaCl in H_2O

[1]SG, step-gradient.
$CHCl_3$, chloroform; MeOH, methanol; EtOH, ethanol; CH_2Cl_2, dichloromethane; EtOAc, ethyl acetate; Me_2CO, acetone.

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filled with either normal (polar) phases (silica or alumina) eluted with organic solvents, or non-polar phases (Amberlite XAD-2, polyamide or Sephadex LH20) eluted with aqueous mixtures (**Table** 7). Ion-exchange phases (e.g. DEAE-Sephadex) eluted with buffers can be used for polar anionic ecdysteroids (**Figure 4**). The size of the column has to be adapted to that of the sample, with a sorbent-to-sample ratio higher than 50 (w : w), and these methods can be used with very large samples. They represent a rather cheap and reasonably efficient procedure for getting fractions from which ecdysteroids can be crystallized (if present in large amounts) or further purified by HPLC (see below). Step-gradient elution with solvents of increasing strength allows the separation of ecdysteroids over a wide range of polarity.

Disposable Cartridges

Small solid phase extraction (SPE) cartridges containing 0.2–1 g of non-polar HPLC phase allow the clean-up of small samples with a good recovery (**Figure 5**). They can also be used to adsorb ecdysteroids from aqueous media, thus allowing an easy and quantitative recovery of ecdysteroids from organ/cell culture media or from HPLC fractions when a non-volatile buffer is used. Normal-phase SPE cartridges have also been used for the fractionation of biological extracts.

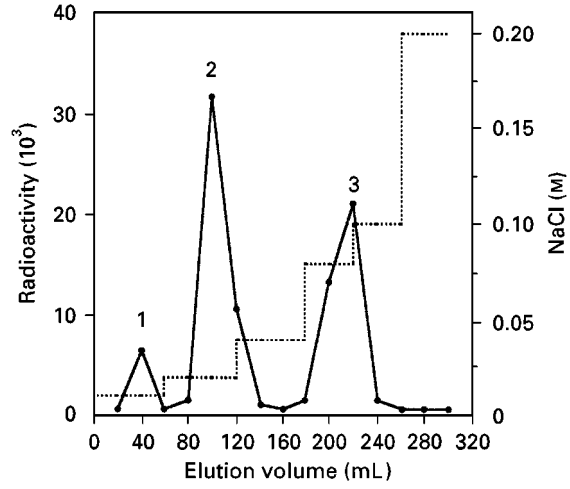

Figure 4 Separation of acidic ecdysteroids from *Manduca sexta* using a DEAE-Sephadex column (6.5 cm long, 2 cm i.d.). Elution was performed with a step-gradient of NaCl. Peak 2 contains ecdysonoic acids, peak 3 contains phosphate conjugates. Redrawn with permission from Lozano R, Thompson MJ, Svoboda JA and Lusby WR (1988) Isolation of acidic and conjugated ecdysteroid fractions from *Manduca sexta* pupae. *Insect Biochemistry* 18: 163–168.

High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) offers a wide range of techniques for analytical and preparative purposes. Co-migration of a compound with a reference ecdysteroid in one (or several) solvent system(s) represents the usual way for identification of the compound. Some common HPLC systems are listed in **Table 8**.

Chromatographic Procedures

Ecdysteroid detection

UV detectors are well-suited to the detection of ecdysteroids, as most ecdysteroids possess a conjugated 7-en-6-one moiety which provides a strongly absorbing chromophore (λ_{max} 242 nm, $\log \varepsilon$ ca. 4). This allows the easy detection of less than 10 pmol amounts.

Diode-array detectors provide information about the absorbance spectrum of all eluted peaks. It can thus be directly checked whether a compound co-migrating with a reference ecdysteroid has a suitable UV spectrum. Good spectra can be obtained with very small amounts (less than 100 ng) of ecdysteroids and such data provide an additional criterion to assess the identity of UV-absorbing peaks.

Fluorescence detectors require the preparation of fluorescent derivatives of ecdysteroids, which may increase the sensitivity of detection by two orders of magnitude when compared with UV. Phenanthrene boronic acid, a reagent specific for α-diols (here the 20,22-diol), and 1-anthroyl nitrile, which reacts with alcohols (here the 2-OH of ecdysteroids), have been used. These reactions, however, are not specific enough for ecdysteroids and they have not been widely adopted.

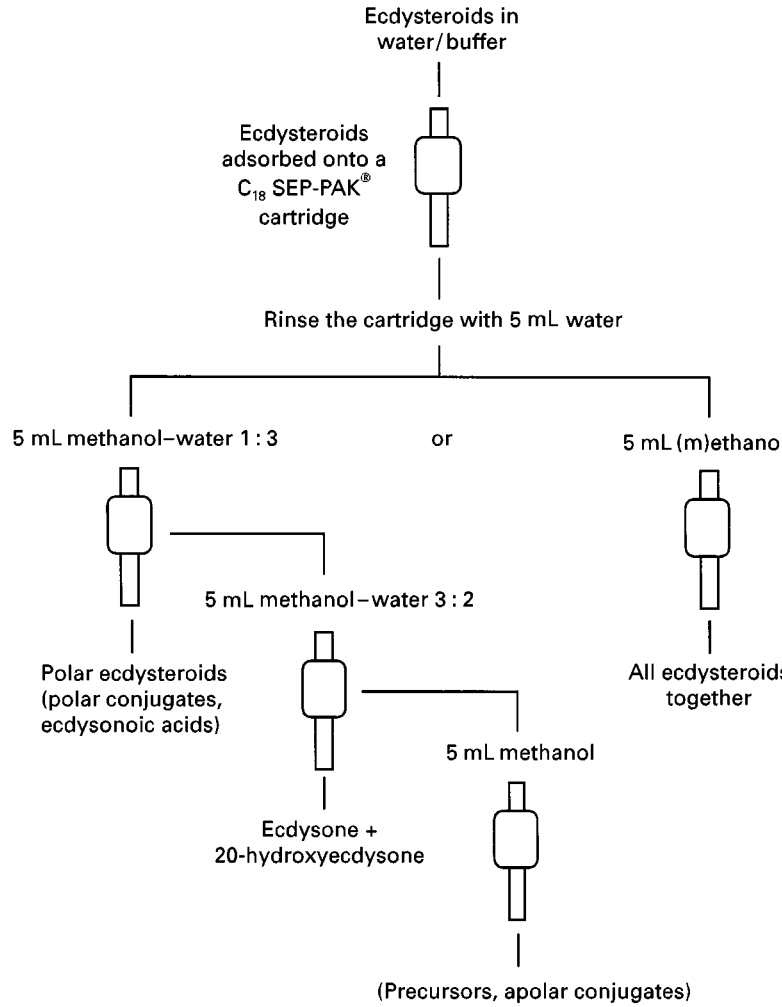

Figure 5 Utilization of reversed-phase cartridges for ecdysteroid purification. The cartridge must be rinsed with 5 mL MeOH then 5 mL water prior to use. Redrawn from Lafont R, Morgan ED and Wilson ID (1994) Chromatographic procedures for phytoecdysteroids. *Journal of Chromatography* 658: 31–53, with permission from Elsevier Science.

Table 8 Chromatographic systems commonly used for the HPLC analysis of ecdysteroids

Mode of chromatography	*Polar*		*Medium*	*Apolar*
	Ionic	*Nonionic*		
Normal-phase (silica, diol, APS, TMS)				
Chloroform/95% ethanol	—		+	+
Chloroform/methanol	—		+	+
Cyclohexane/isopropanol/water	—	—	+	+
Dichloroethane/isopropanol/water	—		+	+
Dichloromethane/tetrahydrofuran/methanol	—		+	+
Dichloromethane/ethanol/water	—		+	+
Dichloromethane/isopropanol/methanol	—		+	+
Dichloromethane/isopropanol/water	—	+	+	+
Dichloromethane/methanol	—		+	+
Dichloromethane/methanol/water/acetic acid	—		+	+
Hexane/ethanol/methanol/acetonitrile	—	—	+	+
Isooctane/isopropanol/water	—	—	+	+
Reverse-phase (C_{18}, C_8, phenyl, etc.)				
Acetonitrile/isopropanol				+
Acetonitrile/isopropanol/water	+	+	+	
Acetonitrile/water	+	+	+	
Acetonitrile/Tris-$HClO_4$, Tris-HCl, Na citrate, TFA 0.1%	+	+	+	
Isopropanol/water	+	+	+	
Methanol/water, Na acetate, Na phosphate	+	+	+	
Methanol				+
Ion-pair				
Acetonitrile/cetrimide-phosphate	+			
Methanol/tetrabutylammonium	+			
Ion-exchange				
Ammonium acetate	+	—	—	—

—, does not apply. APS, aminopropyl silane; TFA, trifluoroacetic acid; TMS, trimethylsilane.

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Radioactivity monitoring provides a direct comparison with UV absorbance, and this easily allows one to make correspondence between the radioactive peaks and unlabelled reference compounds added in the sample before injection. They are currently used for metabolic studies.

Mass spectrometry (MS) gives important structural information. The interfacing problems between HPLC and MS have been overcome in recent years and this technique will undoubtedly develop in the future. An example of HPLC-MS applied to an ecdysteroid-containing plant extract is given in **Figure 6**.

Nuclear magnetic resonance spectrometry can also be used on-line to identify ecdysteroids. This requires rather expensive deuterated HPLC solvents and the method is only suitable for plant extracts where ecdysteroid concentrations are high enough. An example of the use of this emerging technology in a plant extract is shown in **Figure 7**.

Off-line procedures may be used to improve the sensitivity and/or selectivity of ecdysteroid detection (or to identify ecdysteroids after preparative HPLC purification). These analytical methods include chiefly immunoassays (RIA, EIA) and also several recently designed *in vitro* bioassays.

Normal-phase systems Normal-phase HPLC systems generally use silica columns (sometimes polar-bonded columns) and, for example, dichloromethane–isopropanol–water mixtures. Non-polar ecdysteroids (such as esters or precursors) can be separated with a 125 : 15 : 1 (v/v/v) mixture, medium-polarity compounds (such as ecdysone and 20-hydroxyecdysone) with a 125 : 30 : 2 mixture, and more polar (but non-ionic) ecdysteroids (such as 26-hydroxyecdysteroids and glucosides) with 125 : 40 : 3 or 100 : 40 : 3 mixtures.

Dichloromethane-based solvents strongly absorb UV and do not allow UV spectra to be obtained with diode-array detectors, nor are they well suited to in-line radioactivity monitoring (due to their quenching properties). These problems do not exist with

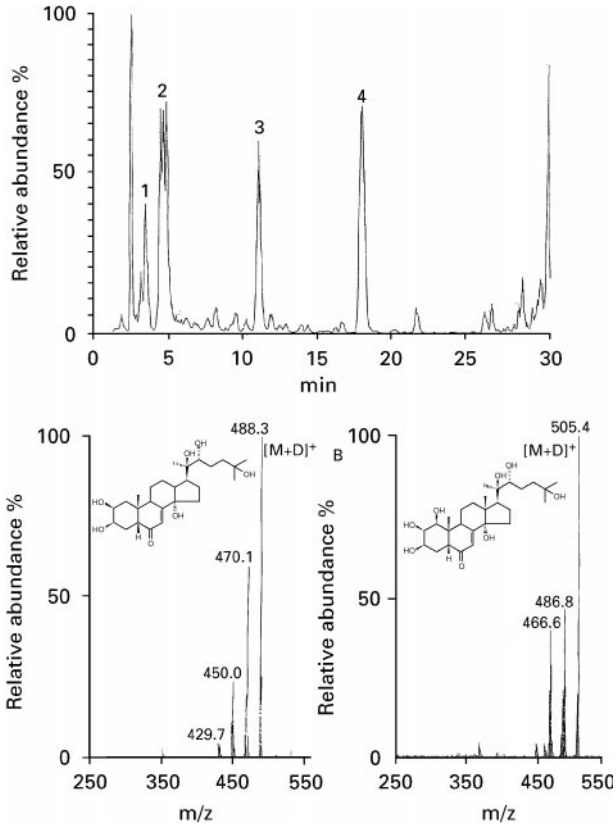

Figure 6 Reversed-phase HPLC-MS total ion current trace (upper) of an extract of *Silene otites*. Peak 1, integristerone A; 2, 20-hydroxyecdysone; 3, 2-deoxy-20-hydroxyecdysone; 4, 2-deoxyecdysone. The mass spectra (lower) are of 20-hydroxyecdysone (left) and integristerone A (right). Structures in insets to mass spectra. After Wilson ID, Lafont R, Shockcor JP *et al.* (1999) High-performance liquid chromatography coupled to nuclear magnetic resonance spectroscopy and mass spectrometry applied to plant products: Identification of ecdysteroids from *Silene otites*. *Chromatographia* 49: 374–378, reprinted with permission.

cyclohexane-based mixtures, however, the poor solubility of ecdysteroids in these mixtures causes some problems for the analysis of polar ecdysteroids and also for preparative purposes.

Polar-bonded columns (e.g. -diol, -polyol or -aminopropylsilane, APS) can also be used. Diol-bonded columns used with extracts of the phasmid *Carausius morosus* allowed the separation of a wide array of polar and non-polar metabolites, whereas the APS phase has provided efficient separations of mixtures containing 3α-OH, 3β-OH and 3-oxo ecdysteroids. One major interest in such polar-bonded columns is that solvent gradients can be used, whereas lengthy re-equilibration times would be the case with silica columns.

Reversed-phase systems Reversed-phase HPLC on C_{18}-bonded columns eluted with methanol–water mixtures provides the most widely used system. Acetonitrile–water (acetonitrile–buffer) mixtures are more efficient, especially when polar conjugates and/or ecdysonoic acids are present. Adequate systems have been designed for polar and apolar metabolites, which may both be present within the same sample. Surprisingly, apolar fatty acyl esters of ecdysteroids are not eluted with pure acetonitrile, whereas they are with methanol, despite the fact it is a more polar solvent (the same is true for cholesterol).

In the case of polar (ionizable) metabolites, it may be of interest to use different pHs, which will result in modified retention times, while uncharged ecdysteroids will retain the same elution time. Moreover, this gives an easy access to the pK_a value of ionizable groups, which is of interest for the characterization of conjugates.

Ion-exchange chromatography Anion-exchange columns are used for the purification of polar conjugates. They represent an efficient method, complementary to reversed-phase HPLC. However, using two different pHs (e.g. pH 7.5, then 2.5) with HPLC, provides an equivalent opportunity for obtaining pure ionizable conjugates, e.g. phosphate esters.

Various Aims of HPLC

Analytical and preparative HPLC Columns of different sizes with the same packing are available, therefore it is very easy to scale up any chromatographic separation. Maximum sample load is related to the amount of stationary phase in the column. In

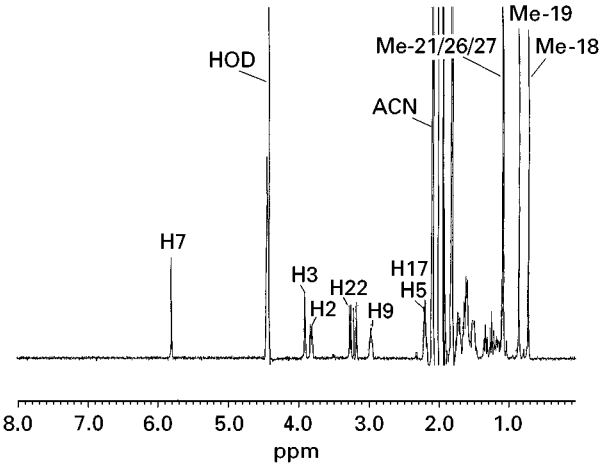

Figure 7 Stopped-flow HPLC-NMR spectrum of 20-hydroxyecdysone following reversed-phase HPLC on a C_{18}-bonded column with D_2O acetonitrile as mobile phase. (Reproduced with permission from Wilson ID, Morgan ED, Lafont R and Wright B (1998) High-performance liquid chromatography coupled to nuclear magnetic resonance spectroscopy. Applications to the ecdysteroids of *Silene otites*. *Journal of Chromatography A* 799: 333–336).

favourable cases, samples up to the milligram range can be run on analytical columns. Larger amounts, of course, require wide-bore columns. It may happen that some compounds that are baseline resolved when loading is low, are not well separated with a larger load. This is, for instance, observed with the inokosterone/20-hydroxyecdysone pair run on a semi-preparative column (Zorbax®-SIL, 9.6 mm i.d.), even when less than 0.5 mg is injected.

Quantitative analyses HPLC has been used for the direct quantification of individual ecdysteroids in biological samples. For animal extracts, this requires a high sensitivity because of the low concentrations present and adequate sample clean-up. The most reliable quantification is obtained if an internal standard is added before sample purification. Many phytoecdysteroids can be used as internal standards. The choice must be made after a preliminary run of a representative sample in order to select a compound that does not co-migrate with major impurities and of course differs from the ecdysteroids present in the biological extract.

Selectivity in HPLC

Both isocratic reversed-phase and normal-phase systems can separate rather complex ecdysteroid mixtures, and the use of solvent gradients increases the

Table 9 HPLC data on a set of ecdysteroids using several chromatographic systems

Compound	*DIW*	*CIW*	*AW*	*MW*	*IW*
2-Deoxy-20-hydroxyecdysone	13.9	11.7	12.5	11.8	14.8
2-Deoxyecdysone	9.5	8.9	31.9	22.6	44.1
3-Dehydro-20-hydroxyecdysone	9.2	16.2	6.5	5.8	
3-Epi-20-hydroxyecdysone	25.7	19.6	5.6	5.4	
5α-2-Deoxy-20-hydroxyecdysone	13.6	13.6	11.6	10.7	13.0
5α-2-Deoxyecdysone	9.2	10.0	30.2	21.7	38.3
5α-20-hydroxyecdysone	24.9	24.1	4.9	4.8	5.0
5α-Ecdysone	15.0	16.2	9.3	7.3	9.9
20-Hydroxyecdysone	**28.9**	**21.3**	**5.2**	**5.4**	**6.0**
20-Hydroxyecdysone 2-acetate	10.7	13.8	14.6	11.3	
20-Hydroxyecdysone 3-acetate	11.3	14.9	10.4	7.7	
20-Hydroxyecdysone 22-acetate	17.3	17.3	12.0	7.6	
20-Hydroxyecdysone 25-acetate	8.7	11.5	18.0	9.5	
20,26-Dihydroxyecdysone	89.9	47.1	3.7	3.7	4.3
22-Deoxy-20-hydroxyecdysone	21.7	15.3	13.5	11.7	18.5
22-Epi-20-hydroxyecdysone	50.0	25.8	4.6	4.7	
22-Oxo-20-hydroxyecdysone	16.8	15.1	12.4	9.8	
24-Epi-makisterone A	16.8	15.5	7.8	6.9	
25-Deoxyecdysone	6.1	6.4	102.9	32.7	
Abutasterone	35.5	27.1	4.4	4.8	5.0
Ajugalactone	7.2	20.2	14.5	7.5	
Cyasterone	9.9	14.5	8.1	5.8	
Ecdysone	**18.8**	**15.1**	**9.7**	**8.4**	**13.0**
Gerardiasterone	40.6	27.9	5.5	6.0	
Integristerone A	38.7	28.8	4.2	4.4	5.0
Makisterone A	20.5	17.7	7.0	6.9	
Makisterone C	11.3	11.3	16.8	12.9	
Polypodine B	18.8	21.5	5.4	5.4	5.5
Ponasterone A	6.6	7.9	32.8	17.5	48.8
Poststerone	9.0	13.3	9.3	6.4	
Rubrosterone	8.7	13.3	5.6	4.3	
Sidisterone	9.6	26.0	12.2	6.3	
Stachysterone C	7.2	8.1	22.7	13.2	
Turkesterone	89.9	36.4	4.4	4.1	3.7
$\triangle^{24,25}$-25-Deoxyecdysone	6.4	6.8		24.3	
$\triangle^{24,28}$-Makisterone A	13.6	13.6	8.0	7.5	
$\triangle^{25,26}$-25-Deoxyecdysone	6.4	6.8		21.1	
$\triangle^{9,11}$-20-Hydroxyecdysone	7.2	7.7		14.1	
$\triangle^{9,11}$-Ecdysone	22.5	16.8		7.2	

Analytical columns were either a Zorbax-Sil column (DuPont), 25 cm × 4.6 mm i.d., eluted with DIW (dichloromethane–isopropanol–water, 125 : 30 : 2) or CIW (cyclohexane–isopropanol–water, 100 : 40 : 3) or a Spherisorb 5ODS2 (Biochrom), 25 cm × 4.6 mm i.d., eluted with water + 1‰ (final concentration) trifluoroacetic acid and either 23% acetonitrile (AW), 50% methanol (MW), or 18% isopropanol (IW). Flow rate was 1 mL min^{-1} in every case.

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power of these systems. In order to draw up general rules, it is necessary to analyse the HPLC behaviour of numerous ecdysteroids differing by single or multiple modifications, and to use a set of different HPLC systems. A set of such data are given in **Table 9**, and the corresponding conclusions are given in **Table 10**.

Selectivity in NP-HPLC

Columns NP-HPLC columns are packed with either silica or polar-bonded silica. The use of diol-bonded material instead of silica does not introduce large changes; retention times vary, but the elution order

Table 10 Effects of individual changes (with reference to the 20E molecule) on the chromatographic behaviour of ecdysteroids as analysed using various chromatographic systems

Change	*Effect on capacity factor (k′)*			
	Normal-phase		*Reversed-phase*	
	DIW	*CIW*	*MW*	*AW*
Change of the number of -OH groups				
+ 1, 11, 24, 26	++(1 < 24 < 11 = 26)	+(24 < 1 < 11 < 26)	−(26 < 11 < 1 < 24) −	−(26 < 1 < 11 = 24)
+ 5	−	=	(−)	(+)
− 2, 20, 22, 25	−(25 < 2 < 20 < 22)	−(25 < 2 < 20 = 22)	+(20 < 2 = 22 < 25)	+(20 < 2 < 22 ≪ 25)
Change of stereochemistry at C-5 (5β → 5α)				
If 2-OH present	−	+	(+)	(−)
If 2-OH absent	=	+	=	(−)
Change of stereochemistry at C-22	++	+	−	−
Change at C-3				
3β-OH → 3-oxo	−−	−	(+)	+
3β-OH → 3α-OH	−	−	=	(+)
Presence of substituents at C-24				
24-Me	−	−	+	+
24-Et	−−	−−	++	++
Conjugation of 20E				
Monoacetates	−−(25 < 2 = 3 < 22)	−(25 < 2 < 3 < 22)	+(22 = 3 < 25 < 2)	++(3 < 22 < 2 < 25)
Monoglucosides	++(22 < 25 < 2 = 3)	Not tested	+(22 < 25 < 2 = 3)	−(22 < 25 < 2 = 3)
Presence of double bonds				
9, 11	(+)	(+)	−	−
25, 26	+	−	−	−
24, 25	+	+	−	−
Presence of a lactone on the side-chain (cyasterone vs. 20E)	−−	(−)		
Side-chain cleavage products				
Poststerone (C_{21})	−−	−−	+	+
Rubrosterone (C_{19})	−−	−−	−	−

Changes of the *k′* value are described as follows: −− , strong reduction; −, reduction; (−), weak reduction; = , almost no change; (+), weak increase; +, increase; ++ , large increase. Numbers in parentheses refer to the positions concerned, and the molecules are classified according to increasing *k′* values. DIW, dichloromethane–isopropanol–water; CIW, cyclohexane–isopropanol–water; MW, methanol–water; AW, acetonitrile–water.

usually remains the same. Aminopropyl (APS) columns may interact specifically with 3-oxoecdysteroids and therefore introduce a different selectivity. C_2-bonded phases behave like weakly active silicas and give very symmetrical peaks; they may be of interest for very polar non-ionic ecdysteroids.

The retention times obtained with silica columns may decrease upon prolonged use, because water-containing solvents slowly deactivate the column. A reactivation cycle with anhydrous solvents: alcohol, dichloromethane, hexane, dichloromethane (50–100 mL each) allows an almost complete recovery of initial retention times.

Solvents UV monitoring of the HPLC effluent precludes the use of solvents such as ethyl acetate, benzene or acetone (such limitations were not encountered with TLC). The solvent is usually based on a chlorinated hydrocarbon (dichloro(m)ethane, chloroform) modified with an alcohol (methanol, ethanol or isopropanol). Water added just below saturation reduces peak tailing.

Dichloromethane- and cyclohexane-based mixtures display a different selectivity, as shown in **Figure 8**. Selectivity changes are particularly impressive when considering the pair $5\alpha/5\beta$, or compounds bearing a lactone ring on the side-chain. As cyclohexane-based mixtures are highly viscous, the working pressures may exceed 100 bar with analytical columns run at a flow-rate of 1 mL min^{-1}. Increasing temperatures to 50°C overcomes this drawback and results in about a 40% decrease of working pressure without affecting the separation.

NP-HPLC is rather inefficient for the separation of compounds with or without extra double bonds, whereas RP-HPLC allows their easy resolution.

Selectivity in RP-HPLC

Columns Many different types of column are available, which differ by the type (C_6, C_8, C_{18}, C_{22}, phenyl, CN, etc.) and extent (percentage of the carbon load) of bonding, and also by the porosity (6–30 nm) of the silica used. All these parameters influence the selectivity of the column, thus, ensuring reproduction of a separation described in the literature requires the same type of column. C_{18} (or ODS) bonded silicas are the most widely used column packings.

Solvents The most common RP-HPLC solvents contain water and either methanol or acetonitrile, although other organic modifiers (ethanol, isopropanol, butanol, etc.) can be used. Using a buffer instead of water, or adding 0.1% (v/v) trifluoroacetic acid often results in much improved separations, especially when ionizable ecdysteroids are present.

The relative retention of ecdysteroids differing by a single -OH group varies with the organic solvent of the mobile phase. Extra -OH groups generally increase the polarity; their effect depends both on their position in the molecule (**Figure 9**) and on the solvent system used.

Isopropanol–water mixtures provide the best separations for $5\alpha/5\beta$ pairs. Methanol is particularly efficient towards extra double bonds and gives a base-

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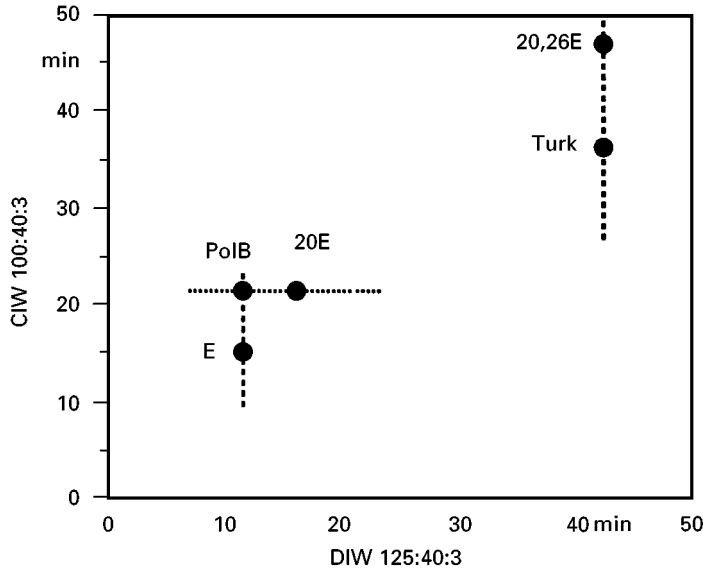

Figure 8 An example of selectivity in NP-HPLC. E, ecdysone; 20E, 20-hydroxyecdysone; 20,26E, 20,26-dihydroxyecdysone; PolB, polypodine B; Turk, turkesterone. CIW, cyclohexane–isopropanol–water; DIW, dichloromethane–isopropanol–water.

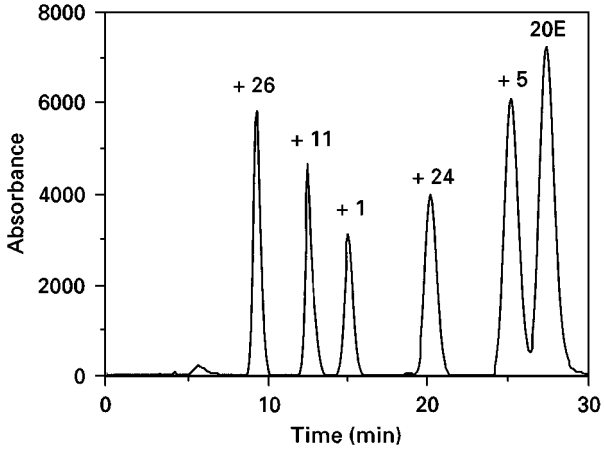

Figure 9 Separation of reference ecdysteroids by RP-HPLC (column Spherisorb 5ODS2, 250 mm × 4.6 mm, solvent 35% methanol in water, isocratic, flow-rate 1 mL min^{-1}). The numbers indicate the position of the additional –OH group by reference to 20-hydroxyecdysone (20E).

line separation of 24,25-ene and 25,26-ene pairs. Higher temperatures increase efficiency, decrease pressure and result in a reduction of k with only limited effects on selectivity.

Supercritical Fluid Chromatography (SFC)

Supercritical carbon dioxide is a non-polar fluid and therefore SFC is more or less equivalent to normal-phase HPLC. The eluting power is increased by adding methanol to the fluid. Various types of packed columns can be used (**Table 11**), including silica or bonded silica; bonded silica is less retentive as is observed with NP-HPLC. Alternatively, fused silica capillary columns can be used.

A major advantage of SFC over HPLC is shorter retention times. The compounds elute as very sharp peaks and the sensitivity of detection is increased accordingly. A second advantage is the high transparency of carbon dioxide in the infrared (IR), which allows the use of FT-IR detectors. Furthermore, SFC is compatible with both flame ionization detection (the universal detection used with GC) and mass spectrometry (interfacing causes far fewer problems than with HPLC). No derivatization is required, and this represents an important advantage over GC. SFC appears, therefore, to be an interesting compromise, but its use has been up to now limited to plant extracts containing particularly high concentrations of ecdysteroids. The easy removal of carbon dioxide after fraction collection represents an advantage for preparative uses. An example of the separation of ecdysteroids by SFC is given in **Figure 10**.

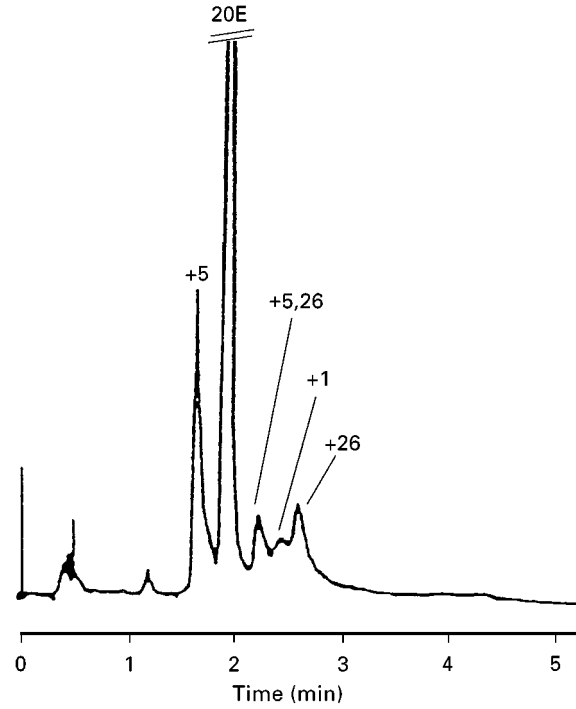

Figure 10 SFC of ecdysteroids on 5 μm-cyanopropyl-bonded silica gel with carbon dioxide–methanol (9 : 1) as mobile phase at 3 mL min^{-1}, 60°C and 290 bar (sample from *Silene otites*). (Reproduced from Raynor MW, Kithinji JP, Bartle K *et al.* (1989) Packed column supercritical-fluid chromatography and linked supercritical-fluid chromatography–mass spectrometry for the analysis of phytoecdysteroids from *Silene nutans* and *Silene otites*. *Journal of Chromatography* 467: 292–298, with permission from Elsevier Science.)

Table 11 Some SFC systems used for ecdysteroid analysis

Column	*Solvent system*
Normal phase (silica)	
Hypersil 5 μm (10 cm × 4.6 mm i.d.)	CO_2–MeOH (4 : 1) 300 bar, 80°C, 4 mL min^{-1}
Bonded phases	
Spherisorb-CN 5 μm	CO_2 or CO_2–MeOH (9 : 1)
Spherisorb-ODS2 5 μm	CO_2 or CO_2–MeOH (9 : 1)

See Lafont *et al*. (1994b) for more details.

Gas Chromatography

Gas chromatography (GC) was first developed for ecdysteroids during the early 1970s. Despite the advantages of GC (especially in combination with the electron capture detector), concerning sensitivity and specificity compared with other techniques, its use for ecdysteroids has been limited. As a majority of biosynthetic intermediates of the ecdysteroids are involatile, it is necessary to convert them into volatile trimethylsilyl ether derivatives. Derivatization is usually performed with either trimethylsilylimidazole (TMSI) or *N*,*O*-bis-trimethylsilyltrifluoroacetamide, although other more specialized derivatives have been used. Complete derivatization is not always obtained and a single ecdysteroid can give rise to several chromatographic peaks.

Not all ecdysteroids are suitable for GC even after silylation. Silylation of compounds such as cyasterone (which contains a lactone in the side-chain) can result in a variety of derivatives characterized either by long retention times or poor peak shapes. Some ecdysteroid conjugates such as coumarate esters, appear to break down to the free ecdysteroid during the silylation procedure.

Chromatographic Systems

Chromatography was first performed with packed columns (1–3 m long, 2 mm i.d.) containing nonpolar stationary phases such as OV-1 and OV-101 coated on Gas Chrom Q and Gas Chrom P. Fused-silica columns (10–25 m long, 0.22 mm i.d.) now provide much better separations. A typical operation temperature for the columns is 280°C.

Flame ionization detectors (FID) allow detection limits in the nanogram range. Electron-capture detection (ECD) provides the best available sensitivity among chromatographic methods (5 pg), and GC coupled with MS gives structural information with as few as 0.1 ng of ecdysteroids (particularly important with animal extracts). However, the need for derivatization is such a drawback that this powerful method is seldom used for ecdysteroids nowadays.

Conclusion

HPLC is the most widely used method for the separation of ecdysteroids. At the present time, hyphenated techniques (HPLC-MS and HPLC-NMR) are developing and will provide particularly powerful tools for the analysis of ecdysteroid-rich (plant) samples in the future.

See Colour Plate 80.

See also: **II / Chromatography: Gas:** Derivatization; Detectors: Selective. **Chromatography: Liquid:** Countercurrent Liquid Chromatography; Derivatization; Detectors: Mass Spectrometry; Detectors: Ultraviolet and Visible Detection; Fluorescence Detectors in Liquid Chromatography; Nuclear Magnetic Resonance Detectors. **Chromatography: Thin-Layer (Planar):** Densitometry and Image Analysis; Layers; Mass Spectrometry; Modes of Development: Conventional; Modes of Development: Forced Flow Over Pressured Layer Chromatography and Centrifugal; Spray Reagents. **Extraction:** Analytical Extractions; Multistage Countercurrent Distribution. **III / Immobilised Boronic Acids: Extraction. Natural Products:** Liquid Chromatography – Nuclear Magnetic Resonance. **Steroids:** Liquid Chromatography and Thin-Layer (Planar) Chromatography; Gas Chromatography; Supercritical Fluid Chromatography.

Further Reading

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